Notes

Oxathiaphospholane Method of Stereocontrolled Synthesis of Diribonucleoside 3′,5′-Phosphorothioates

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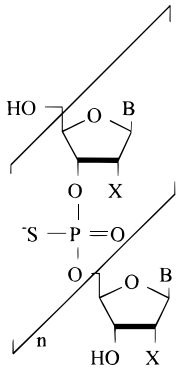
Oligo(deoxyribonucleoside phosphorothioate)s (**1**) (Figure 1), possessing a chiral phosphorothioate function in place of each phosphodiester linkage, are the analogues of natural oligonucleotides most widely employed for inhibition of biosynthesis of "unwanted" proteins by the so-called "antisense strategy".[1] A novel *oxathiaphospholane* approach has recently been elaborated in our laboratory,[2] allowing the synthesis of **1** in a stereocontrolled manner, with predetermined absolute configuration at each internucleotide phosphorothioate center.

An increased interest in the stereocontrolled chemical synthesis of oligo(ribonucleoside phosphorothioate)s (**2**)[3] prompted us to study the adaptation of the oxathiaphospholane method to the requirements of the solid-phase synthesis of **2**. Thus, 5′-*O*-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)ribonucleosides (**3a**−**d**) were treated with 2-(*N*,*N*-diisopropylamino)-1,3,2-oxathiaphospholane[2] and tetrazole, followed by elemental sulfur, giving in satisfactory yield 5′-*O*-DMT-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(2-thiono-1,3,2-oxathiaphospholanyl)ribonucleosides (**4a**−**d**) (Scheme 1). Standard acyl groups were used to protect the reactive amino functions of adenine (benzoyl), cytosine (benzoyl) and guanine (isobutyryl). Compounds **4a**−**d** were isolated by column chromatography as diastereomeric mixtures and were characterized by ^{31}P NMR and FAB-MS. The separation of **4a**−**d** into individual diastereomers (fast- and slow-migrating) was achieved by careful column chromatography in specially designed eluent systems. The experimental conditions are given in Table 1.

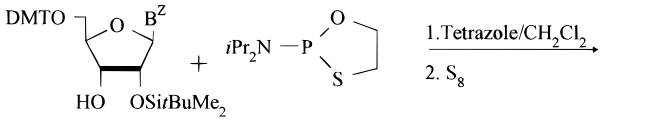
The oxathiaphospholane monomers were reacted with the 5′-hydroxyl group of appropriately protected ribonucleosides under conditions elaborated for the deoxyribonucleoside series.[2] In a preliminary experiment **4a** (mixture of diastereoisomers) was shown to react with 2′,3′-diacetyluridine, on a 25 μmol scale, in acetonitrile containing an equimolar amount of DBU. The reaction was completed within 3 h at room temperature (^{31}P NMR control), and after standard deprotection, diuridine 3′,5′-phosphorothioate ($U_{PS}U$) was isolated in high yield by reversed-phase high performance liquid chromatography (RP HPLC) and identified by ^{31}P NMR and electrospray ionization mass spectrometry (ESI MS). The relative amount of each isomer was identical to that in starting **4a**. The same procedure was repeated with the separated diastereoisomers, **4a**-fast and **4a**-slow, giving in each case the dinucleotide $U_{PS}U$ (as confirmed by ESI MS) of opposite configuration at phosphorus. Thus, **4a**-fast (dp 99.2%) gave $U_{PS}U$ with a HPLC t_R = 10.40 min (dp 99%).



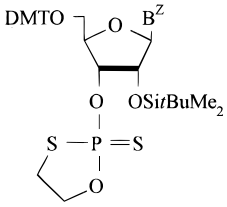
1, X=H; B=Ade, Gua, Cyt, Thy
2, X=OH; B=Ade, Gua, Cyt, Ura

Figure 1.

Scheme 1



3a, B^z= Ura
3b, B^z= Cyt^{Bz}
3c, B^z= Ade^{Bz}
3d, B^z= Gua^{iBu}



4a, B^z= Ura
4b, B^z= Cyt^{Bz}
4c, B^z= Ade^{Bz}
4d, B^z= Gua^{iBu}

Table 1. Separation of Oxathiaphospholane Monomers 4a−b into Individual Diastereoisomers

isomer		eluent	R_f[a]	δ ^{31}P NMR (CD_3CN)
4a	fast	benzene:butyl acetate	0.254	106.59
	slow	(2:1, v/v)	0.210	108.16
4b	fast	benzene:butyl acetate	0.333	106.56
	slow	(1:1, v/v)	0.272	107.86
4c	fast	hexane:ethyl acetate	0.246	106.07
	slow	(1:1, v/v)	0.229	107.43
4d	fast	chloroform:acetone:benzene	0.338[b]	106.65
	slow	(50:1:1, v/v/v)	0.297[b]	107.95

[a] HPTLC Plates 60 F_{254} (Merck). [b] After double development.

(1) Cohen, J. S. In *Antisense Research and Applications*, (Crooke, S. T., Lebleu, B., Eds.; CRC Press: Ann Arbor, **1993**; p 205.

(2) (a) Stec, W. J.; Grajkowski, A.; Koziołkiewicz, M.; Uznański, B. *Nucleic Acids Res.* **1991**, *19*, 5883. (b) Stec, W. J.; Wilk, A. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 709. (c) Stec, W. J.; Grajkowski, A.; Kobylańska, A.; Karwowski, B.; Koziołkiewicz, M.; Misiura, K.; Okruszek, A.; Wilk, A.; Guga, P.; Boczkowska, M. *J. Am. Chem. Soc.* **1995**, *117*, 12019.

(3) (a) Burgers, P. M. J.; Eckstein, F. *Biochemistry* **1979**, *18*, 592. (b) Leśnikowski, Z. J. *Nucleosides Nucleotides* **1992**, *11*, 1621. (c) Almer, H.; Stawiński, J.; Strömberg, R.; Thelin, M. *J. Org.Chem.* **1992**, *57*, 6163. (d) Almer, H.; Stawiński, J.; Strömberg, R. *J. Chem. Soc., Chem. Commun.* **1994**, 1459. (e) Stawiński, J.; Strömberg, R.; Thelin, M.; Westman, E. *Nucleic Acids Res.* **1988**, *16*, 9285.

S0022-3263(96)00811-0 CCC: $12.00

Scheme 2[5]

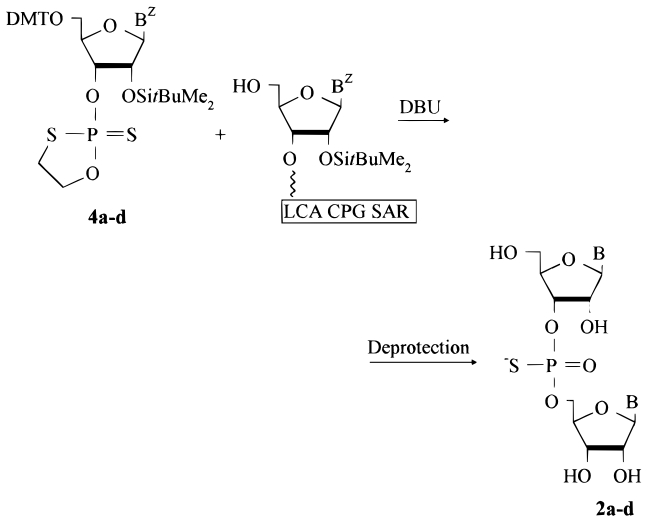


Table 2. Protocol for the Manual Synthesis of Diribonucleoside 3′,5′-Phosphorothioates 2 by the Oxathiaphospholane Approach

entry	step (reagent of solvent)	volume (mL)	time (min)
1	detritylation (3% dichloroacetic acid in CH_2Cl_2)	3	2
2	wash (CH_3CN)	10	3
3	drying (argon)		3
4	drying (vacuum line)		10
5	condensation [0.2 M solution of **4** in CH_3CN (100 μL) + 1 M solution of DBU in CH_3CN (50 μL), premixed]	0.15	13
6	wash (CH_3CN)	10	3

Its absolute configuration was assigned by hydrolysis with snake venom phosphodiesterase (svPDE) and nuclease P1 (nP1), nucleolytic enzymes of known stereospecificity toward internucleotide phosphorothioate linkages.[3a,d] The **4a**-fast-derived diuridine 3′,5′-phosphorothioate was found to be easily hydrolyzed with nP1 and was completely resistant to svPDE, allowing us to assign the (S_P)-configuration at the internucleotide phosphorus atom.[3a,d] The reaction of **4a**-slow (dp 97.8%) gave a mixture of diastereoisomers of $U_{PS}U$, containing predominantly (95.6%) the isomer with the shorter HPLC t_R (9.53 min). Its (R_P)-configuration at phosphorus was confirmed by complete hydrolysis with svPDE into uridine and uridine 5′-phosphorothioate, and resistance toward hydrolysis with nP1.[3a,d]

The stereocontrolled synthesis of diribonucleoside (3′,5′) phosphorothioates by the oxathiaphospholane method was studied further using separated diastereomers of **4a**−**d** and 5′-OH nucleoside bound to the CPG type solid support *via* a DBU-resistant sarcosine-containing linker (LCA CPG SAR)[4] (Scheme 2). Reactions were performed manually on a 1 μmol scale with 20 μmol of **4** and 50 μmol of DBU. The synthesis protocol is presented in Table 2. According to this protocol, eight homodiribonucleoside phosphorothioates ((S_P)- and (R_P)-diastereoisomers of $U_{PS}U$, $C_{PS}C$, $A_{PS}A$, and $G_{PS}G$) were synthesized starting from the individual diastereoisomers of **4a**−**d**. After the final detritylation, cleavage from the support and base deprotection were achieved by incubation of the support-bound dinucleotide with 28% aqueous ammonia diluted with ethanol (3:1, v/v, 1 mL) at r.t. for 16 h. The removal of the silyl protecting group was accomplished by further treatment of the crude product with a 1 M solution of triethylammonium fluoride in THF at r.t. for 16 h. Under these conditions $O^{3'} \rightarrow O^{2'}$ migration of the phosphorothioate moiety, as earlier reported by Stawiński *et al.*,[3e] was observed (HPLC control) to the extent of 2%. The deprotected dinucleotides **2a**−**d** were isolated by preparative HPLC. The results are summarized in Table 3. The (S_P)- and (R_P)-$U_{PS}U$ (**2a**) obtained from oxathiaphospholane **4a** (fast and slow, respectively) by the solid phase method were chromatographically (HPLC) identical to those prepared in solution. The structure of **2a**−**d** was confirmed by negative ESI MS; for each compound the expected [M − H] ion was observed.

The absolute configuration of the P-chiral diribonucleoside phosphorothioates **2a**−**d** was determined by hydrolysis with svPDE and nP1 as described above for the uridine derivatives. In all cases the fast isomers of **4** (of higher chromatographic mobility) were found to be precursors of dinucleoside (3′,5′) phosphorothioates **2** with the (S_P)-configuration, as determined by their complete resistance toward svPDE and fast hydrolysis by nP1 under conditions described in the literature.[3a,d] The dinucleotides **2** obtained from the slow isomers of **4** were easily hydrolyzed by svPDE, indicating their (R_P)-configuration;[3a,d] however, only for (R_P)-$U_{PS}U$ ((R_P)-**2a**) was complete resistance toward nP1 observed. The (R_P)-isomers of **2b**−**d** were found to be hydrolyzed by nP1 under conditions recommended by other authors,[3d] although at a much slower rate than the corresponding (S_P)-isomers. Thus, for diadenosine derivatives $A_{PS}A$ (**2c**) the (S_P)-isomer was almost completely hydrolyzed (98%) after 2 h, whereas under the same conditions the (R_P)-isomer was hydrolyzed to the extent of 5%. Elongation of the reaction time to 16 h increases the extent of enzymatic hydrolysis of the (R_P)-isomer to 56%. This observation is important since nP1 was proposed by Strömberg *et al.* as a stereoselective "scavenger" of products of "unwanted" chirality resulting from the partially stereoselective synthesis of (*all*-R_P)-oligoribonucleoside phosphorothioates.[3d] Our observation seems to limit the practical application of this enzyme to the "stereopurification" of (*all*-R_P)-oligouridine phosphorothioates. The oligoribonucleoside phosphorothioates containing other nucleobases cannot be "stereopurified" by this approach. It should be stressed, however, that the differences in the rates of hydrolysis of diastereomers of diribonucleoside phosphorothioates are distinct enough to make an unambigeous assignment of absolute configuration at phosphorus.

The knowledge of the absolute configuration of the diribonucleoside (3′,5′) phosphorothioates **2a**−**d** allowed us to assign the chirality at phosphorus of their precursors, **4a**−**d**. We have recently demonstrated[6] that in the deoxyribonucleoside series of oxathiaphospholanes, the corresponding nucleoside 3′-*O*-2-thiono-1,3,2-oxathiaphospholane of the (R_P)-configuration is a "precursor" of (R_P)-dinucleoside (3′,5′) phosphorothioate. Since the presence of a 2′-*O*-silylated substituent should not influence the mechanism of the 1,3,2-oxathiaphospholane ring-opening condensation process and the stereochem-

(4) (a) Brown, T.; Pritchard, C. E.; Turner, G.; Salisbury, S. A. *J. Chem. Soc., Chem. Commun.* **1989**, 891. (b) Lehman, C.; Xu, Y.-Z.; Christoloulos, C.; Tan, Z. K.; Gait, M. J. *Nucleic Acids Res.* **1989**, *17*, 2379.

(5) For support-bound nucleosides, the same protecting groups (*Z*) were used as for **4a**−**d**.

(6) Stec, W. J.; Karwowski, B.; Guga, P.; Misiura, K.; Wieczorek, M.; Błaszczyk, J. *Phosphorus, Sulphur Silicon*, **1996**, *109*−*110*, 257.

Table 3. Stereocontrolled Synthesis of Diribonucleoside Phosphorothioates by the Oxathiaphospholane Approach According to Scheme 2

oxathiaphospholane substrates **4a**−**d**				diribonucleoside 3′,5′-phosphorothioate **2a**−**d**						
					HPLC[b]				molecular weight	
compd	diastereoisomer	dp[a] (%)	support-bound nucleoside B	compd	t_R (min)	yield[c] (%)	dp[c] (%)	phosphorus chirality[d]	calculated[e]	measured[f]
4a	fast	99.2	Ura	$U_{PS}U$ (**2a**)	10.40	97.8	99.0	S_P	566.5	565.6
	slow	97.8			9.53	96.0	95.6	R_P		565.6
4b	fast	100.0	Cyt^{Bz}	$C_{PS}C$ (**2b**)	9.60	76.2	100.0	S_P	564.5	563.7
	slow	99.4			7.98	82.8	98.4	R_P		564.1
4c	fast	98.8	Ade^{Bz}	$A_{PS}A$ (**2c**)	14.52	65.7	98.1	S_P	612.6	611.6
	slow	97.8			12.40	76.5	97.4	R_P		611.8
4d	fast	98.9	Gua^{iBu}	$G_{PS}G$ (**2d**)	12.15	77.8	97.4	S_P	644.6	643.9
	slow	95.9			10.68	70.1	92.2	R_P		643.2

[a] Diastereoisomeric purity as measured from integrated ^{31}P NMR spectra. [b] Linear gradient 0−20% CH_3CN in 0.1 M TEAB in 20 min. [c] Calculated from integrated HPLC chromatograms. [d] Absolute configuration at phosphorus in a predominant diastereoisomer as determined by hydrolysis with svPDE and nP1. [e] The molecular weight calculated for the dinucleotide in a free acid form. [f] *m*/*z* of M − H ions measured by negative ESI MS.

ical result of reactions occurring at the phosphorus center, we can predict the (S_P)-configuration for all **4**-fast and, consequently, the (R_P)-configuration for all **4**-slow at the phosphorus atom.

The comparison of diastereoisomeric purities of **2** with those of starting **4** shows that the syntheses are highly stereoselective. The stereoselectivity, defined as the ratio of dp of **2** divided by the dp of **4**, for most cases was found to be in the range of 98−100%; only in one case (**4d**-slow) was a lower value (96%) observed.

An inspection of Table 3 shows that only in the case of the uridine derivatives are the yields of isolated dinucleotides in the range acceptable for solid-phase oligonucleotide synthesis (96−98%); lower yields (66−83%) were observed for the other compounds. Attempts to increase the yields by extention of the coupling time or increasing the DBU concentration were unsuccessful. Further experiments using the oxathiaphospholane method for solid-phase stereocontrolled synthesis of longer oligo(ribonucleoside phosphorothioate)s are in progress.

Experimental Section

Materials and Methods. The solvents were dried over calcium hydride and distilled before use. All reactions involving trivalent phosphorus compounds were performed under dry argon. Column chromatography and thin layer chromatography (TLC) were performed on 230−400 mesh silica gel and silica gel F 254 plates, respectively (Merck). Dichloromethane:acetone (9:1, v/v) was used as the TLC developing system. Separation of the diastereomeric 5′-*O*-DMT-2′-*O*-TBDMS-3′-*O*-(2-thiono-1,3,2-oxathiaphospholanyl)nucleosides was performed by column chromatography on silica gel 60H using HPTLC plates 60 F 254 (Merck) for analysis.

Reversed-phase high performance liquid chromatography was performed using a PTH C-18 (5 μm) 2.1 × 220 mm column using linear gradient of acetonitrile in 0.1 M triethylammonium bicarbonate (TEAB), pH 7.0. ^1H NMR (300 MHz) and ^{31}P NMR (81 MHz) spectra were referenced to TMS (internal) and 85% H_3PO_4 (external), respectively. FAB mass spectra were recorded at 13 keV (Cs^+ gun), using a 3-nitrobenzyl alcohol matrix.

Electrospray ionization mass spectrometry (ESI MS) was performed on a Finnigan TSQ 700 instrument. 5′-*O*-DMT-2′-*O*-TBDMS-uridine, 5′-*O*-DMT-2′-*O*-TBDMS-N^4-Bz-cytidine, 5′-*O*-DMT-2′-*O*-TBDMS-N^2-iBu-guanosine, and 5′-*O*-DMT-2′-*O*-TBDMS-N^6-Bz-adenosine were purchased from ChemGenes Corp. ^1H-tetrazole, DMAP, and DBU were obtained from Aldrich. Dichloroacetic acid (DCA) was obtained from Riedel-de Haën and distilled before use. Acetonitrile was obtained from Baker. Long chain alkylamine controlled-pore glass 500 Å (LCA CPG) was obtained from Sigma. Fmoc-sarcosine was purchased from Bachem. 5′-*O*-DMT-2′-*O*-TBDMS-*N*-protected ribonucleosides were succinylated by the standard procedure[7] and attached to sarcosinylated LCA CPG as described by Brown *et al.*[4a] The following loadings of derivatized nucleosides were achieved (μmol/g): U, 33.3; N^4-Bz-C, 37.1; N^2-iBu-G, 28.6, and N^6-Bz-A, 40.4.

svPDE (EC 3.1.15.1) was purchased from Boehringer Mannheim. nP1 (EC 3.1.30.1) was obtained from Sigma.

3′-*O*-(2-Thiono-1,3,2-oxathiaphospholanyl)nucleosides 4a−d. Protected nucleoside [5′-*O*-DMT-2′-*O*-TBDMS-uridine, 5′-*O*-DMT-2′-*O*-TBDMS-N^4-Bz-cytidine, 5′-*O*-DMT-2′-*O*-TBDMS-N^2-iBu-guanosine or 5′-*O*-DMT-2′-*O*-TBDMS-N^6-Bz-adenosine] (1 mmol) was mixed with tetrazole (70 mg, 1 mmol), dried for 12 h under high vacuum, and dissolved in methylene chloride (4 mL) distilled from CaH_2. Into the resulting solution stirred at room temperature was added 2-(*N*,*N*-diisopropylamino)-1,3,2-oxathiaphospholane (270 mg, 1.3 mmol) by injection through a rubber septum. Stirring at room temperature was continued until the TLC showed full disappearance of nucleoside substrate (*ca.* 3 h). Elemental sulfur (60 mg) dried under vacuum was added, and the mixture was stirred overnight at room temperature. The solvent was evaporated, and the residue was chromatographed (3 × 15 cm column) using chloroform with a linear gradient of methanol (0 → 3%) to give **4a**−**d** in the form of an amorphous powder. The identity and purity of **4a**−**d** was confirmed by mass spectrometry and ^{31}P NMR (CD_3CN).

4a: yield 95%; TLC R_f 0.56; ^{31}P NMR δ 108.28, 106.69 (41:59);[8] MS (FAB^-) calcd 798.9 (M), found 797.4 (M − H).

4b: yield 99%; TLC R_f 0.65; ^{31}P NMR δ 107.85, 106.56 (46:54);[8] MS (FAB^-) calcd 902.0 (M), found 900.4 (M − H).

4c: yield 99%; TLC R_f 0.69; ^{31}P NMR δ 107.41, 106.06 (43:57);[8] MS (FAB^-) calcd 926.0 (M), found 924.4 (M − H).

4d: yield 88.5%; TLC R_f 0.36; ^{31}P NMR δ 107.92; 106.63 (40:60);[8] MS (FAB^-)calcd 907.8 (M), found 906.4 (M − H).

Separation of the Diastereomers of Oxathiaphospholane Monomers 4a−d. A solution of 400 mg of monomers **4a**−**d** in 1.5 mL of appropriate eluent (Table 1) was applied to a column (6 × 30 cm) containing 90 g of silica gel 60H. The column was eluted with the appropriate eluent (Table 1) and fractions of 10−12 mL were collected. After TLC analysis of each fraction, the fractions enriched in the same diastereomer were combined, concentrated, and rechromatographed. The diastereomeric purity of the separated monomers **4a**−**d** was estimated by ^{31}P NMR (Table 3).

4a-fast: ^1H NMR ($CDCl_3$) δ 0.13 (s, 3H), 0.15 (s, 3H), 0.89 (s, 9H), 3.35−3.48 (m, 2H), 3.52 (dd, 2H, J = 2.26 Hz, 5.29Hz), 3.80 (s, 6H), 4.34−4.36 (m, 1H), 4.47 (t, 1H, J = 4.72Hz), 4.37−4.55 (m, 2H), 5.26 (d, 1H, J = 8.24Hz), 5.30 (dt, 1H, J = 4.72 Hz, 14.56Hz), 6.01 (d, 1H, J = 4.74Hz), 6.85 (d, 4H, J = 8.94Hz), 7.23−7.40 (m, 9H), 7.94 (d, 1H, J = 8.21 Hz).

4a-slow: ^1H NMR ($CDCl_3$) δ 0.10 (s, 3H), 0.16 (s, 3H), 0.90 (s, 9H), 3.44−3.54 (m, 4H), 3.80 (s, 6H), 4.32−4.38 (m, 1H), 4.41 (t, 1H, J = 5.99Hz), 4.44−4.52 (m, 2H), 5.28 (ddd, 1H, J = 2.69

(7) Scaringe, S. A.; Francklyn, C.; Usman, N. *Nucleic Acids Res.* **1990**, *18*, 5433.

(8) Ratio of diastereoisomers by virtue of asymmetry of phosphorus atom.

Hz, 5.30 Hz, 13.19Hz), 5.29 (d, 1H, J = 8.19Hz), 6.85 (d, 4H, J = 8.94Hz), 7.26−7.39 (m, 9H), 7.87 (d, 1H, J = 8.18 Hz).

4b-fast: ^{1}H NMR δ 0.16 (s, 3H), 0.22 (s, 3H), 0.91 (s, 9H), 3.29−3.37 (m, 2H), 3.50 (dd, 1H, J = 2.33 Hz, 11.17Hz), 3.71 (dd, 1H, J = 1.56 Hz, 11.17Hz), 3.82 (s, 6H), 4.29−4.39 (m, 1H), 4.48 (t, 1H, J = 2.58Hz), 4.41−4.51 (m, 2H), 5.19 (ddd, 1H, J = 4.26 Hz, 6.43 Hz, 14.39Hz), 6.04 (d, 1H, J = 2.61Hz), 6.88 (d, 4H, J = 8.79Hz), 7.33 (d, 2H, J = 9.01Hz), 7.29−7.61 (m, 12 H).

4b-slow: ^{1}H NMR δ 0.16 (s, 3H), 0.18 (s, 3H), 0.91 (s, 9H), 3.45−3.50 (m, 2H), 3.52−3.55 (m, 1H), 3.61−3.64 (m, 1H), 3.82 (s, 6H), 4.25−4.35 (m, 2H), 4.40−4.42 (m, 1H), 4.51 (t, 1H, J = 3.82Hz), 5.24 (dt, 1H, J = 4.79 Hz, 13.79Hz), 6.10 (d, 1H, J = 3.70Hz), 6.88 (d, 4H, J = 8.92Hz), 7.33 (d, 2H, J = 8.92Hz), 7.26−7.61 (m, 12H).

4c-fast: ^{1}H NMR δ 0.04 (s, 3H), 0.07 (s, 3H), 0.76 (s, 9H), 3.45 (dd, 1H, J = 3.27 Hz, 11.0Hz), 3.46−3.55 (m, 2H), 3.57 (dd, 1H, J = 3.34 Hz, 10.73Hz), 3.787 (s, 3H), 3.788 (s, 3H), 4.34−4.58 (m, 3H), 5.12 (t, 1H, J = 5.05Hz), 5.41 (ddd, 1H, J = 2.47 Hz, 4.87 Hz, 13.87Hz), 6.15 (d, 1H, J = 6.45Hz), 6.84 (d, 2H, J = 8.99Hz), 6.88 (d, 2H, J = 8.98Hz), 7.37 (d, 2H, J = 8.46Hz), 7.23−7.59 (m, 12H), 8.25 (s, 1H), 8.75 (s, 1H).

4c-slow: ^{1}H NMR δ 0.02 (s, 3H), 0.07 (s, 3H), 0.74 (s, 9H), 3.46−3.59 (m, 4H), 3.78 (s, 6H), 4.39−4.53 (m, 3H), 5.15 (t, 1H, J = 6.85Hz), 5.36 (ddd, 1H, J = 1.85 Hz, 4.76 Hz, 13.21Hz), 6.16 (d, 1H, J = 7.06Hz), 6.83 (d, 2H, J = 8.98Hz), 6.84 (d, 2H, J = 8.99Hz), 7.36 (d, 2H, J = 8.52Hz), 7.22−7.62 (m, 12H), 8.24 (s, 1H), 8.74 (s, 1H).

4d-fast: ^{1}H NMR δ −0.09 (s, 3H), 0.1 (s, 3H), 0.60 (d, 3H, J = 6.88Hz), 0.81 (s, 9H), 0.82 (d, 3H, J = 6.81Hz), 1.34 (sp, 1H, J = 6.80Hz), 3.11 (dd, 1H, J = 3.01 Hz, 10.88Hz), 3.40−3.53 (m, 2H), 3.58 (dd, 1H, J = 1.71 Hz, 10.91Hz), 3.75 (s, 3H), 3.77 (s, 3H), 4.33−4.51 (m, 3H), 5.34 (t, 1H, J = 6.54Hz), 5.51 (ddd, 1H, J = 3.07 Hz, 5.39 Hz, 14.22Hz), 5.72 (d, 1H, J = 6.53Hz), 6.78 (d, 2H, J = 8.94Hz), 6.80 (d, 2H, J = 8.94Hz), 7.22−7.59 (m, 9H), 7.78 (s, 1H).

4d-slow: ^{1}H NMR δ 0.07 (s, 3H), 0.1 (s, 3H), 0.55 (d, 3H, J = 6.86Hz), 0.78 (d, 3H, J = 6.82Hz), 0.81 (s, 9H), 1.25 (sp, 1H, J = 6.87Hz), 3.15 (dd, 1H, J = 2.82 Hz, 10.92Hz), 3.43−3.54 (m, 2H), 3.57 (dd, 1H, J = 1.64 Hz, 10.92Hz), 3.75 (s, 3H), 3.77 (s, 3H), 4.33−4.46 (m, 3H), 5.36−5.44 (m, 2H), 5.72 (d, 1H, J = 6.85Hz), 6.78 (d, 2H, J = 8.97Hz), 6.81 (d, 2H, J = 8.97Hz), 7.21−7.61 (m, 9H), 7.78 (s, 1H).

The Manual Synthesis of Diribonucleoside Phosphorothioates 2a−d. For the synthesis of **2a**−**d** on a 1 μmol scale the protocol described in Table 2 was used. The syntheses were performed in standard DNA synthesis columns (Applied Biosystems). The reagents were introduced by attaching the column to a syringe *via* the appropriate adaptor. After completion of the last step, the dimethoxytrityl group was removed from diribonucleoside 3′,5′-phosphorothioates with a 3% solution of DCA in methylene chloride (3 mL) and the support was washed thoroughly with acetonitrile (10 mL). After drying with argon the support was transferred to a glass vial and treated with a mixture of 28% aqueous ammonia and EtOH (3:1, v/v) (1 mL). The vial was tightly closed and kept for 16 h at room temperature. After this time the sample was cooled down (dry ice/ethanol bath), the ammonia solution was filtered, and the CPG support was washed with ethanol (2 × 500 μL). The combined filtrates were evaporated to dryness. The residue was treated with a 1 M solution of triethylammonium fluoride in THF (1 mL), kept at room temperature for 16 h, and then evaporated to dryness. The purity and identity of **2a**−**d** were checked by RP HPLC (PTH C-18 column, 0.3 mL/min, 0 → 20% CH_3CN in 20 min) and negative ESI MS (Table 3).

Reaction with svPDE. A sample of diastereomerically pure diribonucleoside phosphorothioate **2a**−**d** (0.1 OD unit) was dissolved in 100 μL of 100 mM Tris-HCl buffer (pH 8.5) containing 15 mM $MgCl_2$. Then 0.5 μg of svPDE was added, and the mixture was incubated at 37 °C for 24 h. The sample was heat-denaturated (2 min, 95 °C), centrifuged (10 000 rpm, 10 min), and analyzed on RP HPLC (PTH C-18 column, 0.3 mL/min, 0 → 20% CH_3CN in 20 min).

Reaction with nP1. A sample of diastereomerically pure diribonucleoside phosphorothioate **2a**−**d** (0.1 OD unit) was dissolved in 100 μL of 28.5 mM $(NH_4)_2SO_4$ buffer (pH 5.3) containing 0.5 mM $ZnCl_2$. Then 2 μg of nP1 was added and the mixture was incubated at 37 °C for 2, 4, 8, 16, and 24 h. The aliquots were heat-denaturated (2 min, 95 °C), centrifuged (10 000 rpm, 10 min), and analyzed on RP HPLC (PTH C-18 column, 0.3 mL/min, 0 → 20% CH_3CN in 20 min).

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